Comparison of Protein Profiles during Cotton (*Gossypium hirsutum* L.) Fiber Cell Development with Partial Sequences of Two Proteins

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Two-dimensional polyacrylamide gel electrophoresis was used to compare the protein profiles of cotton (*Gossypium hirsutum* L.) fiber cells at two developmental stages. In the first stage, the cells are elongating and synthesizing primary cell walls [14 days postanthesis (DPA)]; in the second stage, the cells are beginning secondary cell wall synthesis [21 DPA]. Forty-five proteins were found that differed in amount between the two stages; these proteins were characterized by their molecular weights, isoelectric points, and relative quantities at each stage. Two of the proteins that were present in higher amounts in the 21-DPA fiber were further studied by N-terminal amino acid sequencing. One of these proteins was identified as a cytoplasmic malate dehydrogenase on the basis of N-terminal sequence similarity. These results provide a foundation for further studies of the proteins that participate in cotton fiber development.

Keywords: *Gossypium hirsutum L.; Malvaceae; cotton; fiber; two-dimensional electrophoresis; proteins; malate dehydrogenase*

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) fibers are single-celled trichomes that grow from the ovule epidermis and represent the most economically important portion of the cotton crop. Because of more efficient, high-speed-mill processing of the fiber entering the market, further improvements in fiber quality are needed (Meredith et al., 1991). Geneticists have been working to improve fiber quality; however, germplasm with improved fiber quality is often low yielding (Meredith and Bridge, 1971; Scholl and Miller, 1976), indicating a complex relationship between quality and yield. A better understanding of fiber cell physiology is necessary to assist in the improvement of fiber quality.

Growth of fiber cells can be divided into four stages: initiation, elongation, secondary cell wall (SCW) synthesis, and maturation (Jasdanwala et al., 1977). Fiber cells within a boll initiate growth about the same time and continue their development in a nearly synchronous manner (Stewart, 1975). Elongation begins about 1 day postanthesis (DPA) (Stewart, 1975) and continues until 22−26 DPA (Schubert et al., 1973; Meinert and Delmer, 1977). During this elongation, the cells grow to lengths of 1000−3000 times their diameter (diameter $\approx 20\ \mu$m) (DeLanghe, 1986). Overlapping with the elongation stage, deposition of the SCW begins between 16 and 18-DPA (Schubert et al., 1973; Meinert and Delmer, 1977). The cells continue depositing layers of SCW up to 40−50 DPA. Finally, as the carpel dehisces, fiber cells desiccate and become mature fiber.

As the fiber cells progress from the elongation to the SCW stage, a number of proteins have been reported to change. These proteins have been measured by immunological methods (Kloth, 1989; John and Crow, 1992; Dixon et al., 1994), by enzymatic activity (Jasdanwala et al., 1977; Naithani et al., 1981; Basra and Malik, 1983; Wäfler and Meier, 1994), and by binding of cofactors (Amor et al., 1991) and inhibitors (Delmer et al., 1987). For example, Kloth (1989) found that the tubulin proteins increased proportionately with elongation and then remained present during the SCW synthesis. Although informative, the scope of each of these studies is limited to the preselected proteins and their associated metabolism. One approach, which encompasses a larger scope, is to separate proteins simultaneously with two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and determine the differences in protein constituents as the fiber develops.

The use of 2-D PAGE in studying fiber development has not been fully utilized. Graves and Stewart (1988) previously studied fiber development with 2-D PAGE; however, problems associated with secondary metabolites coextracting with proteins resulted in poor resolution. We have utilized methods that overcome these previous limitations and have compared the protein profiles of 14-DPA (midelongation stage) and 21-DPA (early SCW synthesis stage) fiber. With the use of microsequencing (LeGendre et al., 1993), we have begun to identify some of the proteins that change during fiber development.

MATERIALS AND METHODS

Plant Material. Cotton plants (cv. DPL 90ne, Delta and Pine Land, Scott, MS) were grown in an irrigated field near Stoneville, MS, during the summer of 1993. To obtain fiber samples for 2-D PAGE analysis, open flowers (0 DPA) were tagged on July 14, 1993, in each of three replications. Tagged bolls were excised from the plants at 14 or 21 DPA. On the two harvesting dates, the excised bolls were transported to the laboratory and opened, and the locules were removed with gloved hands. The developing fiber was removed from each locule, separated from the seed, placed immediately in liquid nitrogen, and ground to a fine powder with a mortar and pestle; the ground fiber was kept frozen while being transferred to −80 °C storage.

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To obtain fiber samples for cellulose analysis, open flowers (0 DPA) were tagged on July 22, 26, and 30, 1993. Two bolls for each tag date and replication were harvested on August 11, 1993. The developmental ages of these bolls were 12, 16, and 20 DPA. The immature fiber within each sample was separated from the seeds, frozen on dry ice, lyophilized, and ground to a fine powder in a mill. The cellulose content was determined on a dry-weight basis according to the method of Updegraff (1969).

Chemicals and Reagents. Acrylamide, piperazine diacrylamide, ampholytes (5−7 and 3−10), glycine, SDS, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, low molecular weight SDS protein standards, Bio-Rad protein assay reagent, and γ-globulin were obtained from Bio-Rad Laboratories, Hercules, CA. Ultrapure urea, Tris, and phenol were obtained from United States Biochemical Corp., Cleveland, OH. The 1,4-dimethylpiperazine and 50% glutaraldehyde were obtained from Aldrich, Milwaukee, WI. Methanol, ethanol, and silver nitrate were obtained from J. T. Baker, Inc., Phillipsburg, NJ. All other chemicals were obtained from Sigma, St. Louis, MO.

Protein Extraction. The phenol extraction method of Hurkman and Tanaka (1986) was adapted to extract total protein from cotton fiber cells. Immediately after the ground fiber tissue was removed from the −80 °C freezer, the tissue was homogenized with a Polytron (Brinkman Instruments, Inc., Westbury, NY) for 1 min in equal amounts of buffer-saturated phenol (pH >7.4) and extraction buffer [500 mM Tris-HCl (pH 8.65), 50 mM EDTA, 100 mM KCl, and 2% (v/v) β-mercaptoethanol]. For each gram of tissue, 3 mL of phenol and 3 mL of extraction buffer were used. The homogenate was centrifuged for 10 min at 12000*g* at 20 °C to separate aqueous and phenol phases. Discarding the aqueous phase and the pelleted material, the interface and the phenol phase were transferred to another glass centrifuge tube. The phenol phase (containing the protein) was re-extracted three more times by adding a volume of extraction buffer equal to the current phenol volume, vortexing 3 min, centrifuging to separate the phases, and discarding the aqueous phase. After the last re-extraction, the interface and the phenol phase were transferred to a new glass centrifuge tube, and the protein was precipitated by adding 5 or more volumes of 0.1 M ammonium acetate in methanol and storing at −20 °C overnight. The precipitated protein was collected by centrifugation, washed three times by resuspending the pellet in 0.1 M ammonium acetate in methanol, washed once in cold acetone (−20 °C), and dried under vacuum. The protein pellet was solubilized in 1 mL of 0.5% SDS, 9.5 M urea, 150 mM dithiothreitol (DTT), and 0.2% ampholytes (pH 6−8) without heating. After the protein was in solution, an equal volume of 9.5 M urea, 150 mM DTT, 2% Nonidet P-40 detergent, and 2% ampholytes (pH 6−8) was added to the protein solution and mixed (O'Farrell et al., 1977). Samples were centrifuged at 200000*g* for 1 h, divided into aliquots, and stored at −80 °C. Protein concentrations were measured according to the Bradford method (Bio-Rad Laboratories) with γ-globulin as the standard.

2-D Electrophoresis. The 2-D PAGE method utilized piperazine diacrylamide as a cross-linker in both the isoelectric focusing (IEF) and second-dimension gels as described by Hochstrasser et al. (1988). Each protein sample was separated with two different pH ranges. In the first set of gels (set A), the IEF gels contained 2% ampholytes with a pH range of 5−7 and 0.2% ampholytes with a pH range of 3−10. In the second set of gels (set B), the IEF gels contained 2% ampholytes with a pH range of 6−8 and 0.2% ampholytes with a pH range of 3−10. Seventy-five micrograms of protein was loaded on each IEF gel (1.5 mm diameter × 140 mm long). The IEF gels were focused for 1 h at 200 V, for 1 h at 500 V, and for 16 h at 700 V. The second-dimension gels consisted of 9−16% acrylamide gradient gels with the addition of 2 mM sodium thiosulfate; they were polymerized with 1,4-dimethylpiperazine (17 mM) and ammonium persulfate (1.8 mM) to reduce background staining with ammoniacal silver (Hochstrasser and Merril, 1988). After the 2-D electrophoresis was completed, the gels were stained with ammoniacal silver (Hochstrasser et al., 1988).

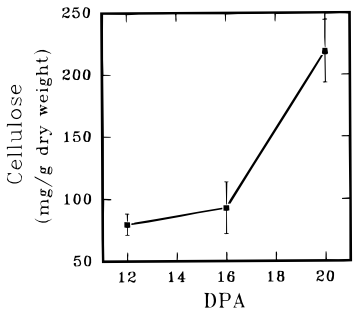

Figure 1. Changes in cellulose content of the fiber cells with time after anthesis.

Analysis of 2-D Gels. Dried gels were analyzed using video densitometry (Model 50C-2D, Bio-Image, Ann Arbor, MI). Protein profiles of 14- vs 21-DPA fiber from each replication were compared with the 2-D Analyzer program ver. 6.0 (Bio-Image). Proteins that were "unique" to one developmental stage were identified and are described under Results. Proteins that were present in both stages were matched and analyzed for differences in quantity. The gels were normalized using a matched ratio algorithm, and the integrated intensities of matched spots were compared. Matched spots with an average 2-fold difference between 14- and 21-DPA gels are discussed under Results.

Protein and DNA Sequencing. To isolate sufficient quantities of each protein for sequencing, additional protein from 21-DPA fiber was extracted. Between 10 and 30 mg of total fiber protein was separated with preparative isoelectric focusing (Rotofor, Bio-Rad) in 8 M urea and 2% ampholytes (pH range 5−7 or 6−8 depending on the protein to be isolated). After the fractions were harvested, the pH of each fraction was determined. The proteins within each fraction were determined empirically by running 2-D PAGE gels. Up to 140 μL of the fraction containing the protein of interest was loaded on IEF gels (3.5 mm diameter × 140 mm long) and electrofocused. The IEF gel was loaded on a 3 mm thick 2-D gel. For electrophoresis of the second dimension, the gel mixture and the electrophoresis buffer were as previously described with one exception: they also contained 100 μM sodium thioglycolate and 50 μM glutathione. 2-D gels were prerun 14−16 h at 50 V. After electrophoresis, proteins within the gel were transferred to a poly(vinylidene fluoride) membrane (Immobilon-P, Millipore, Bedford, MA) using the procedure of LeGendre et al. (1993). We substituted ethanol for methanol in the transfer buffer and staining solutions to facilitate disposal. Proteins on the poly(vinylidene fluoride) membrane were stained with 0.1% Coomassie blue R-250 in 50% (v/v) ethanol for 2 min and then destained in 50% ethanol. Proteins from several blots were pooled and sent to the Biotechnology Core Facility at Kansas State University (Manhattan, KS) for sequencing. The N-terminal acid sequences obtained were compared with known sequences of proteins in the University of Geneva Protein Sequence Data Bank (SwissProt, release 27) with PC/Gene programs (release 6.80, IntelliGenetics, Inc., Mountain View, CA). Additionally, the N-terminal amino acid sequences were compared with nucleic acid sequences in GenBank 84 using the FASTDB program (Brutlag et al., 1990). DNA sequencing was performed using double-stranded DNA templates and dideoxy chain termination techniques with fluorescent primers on the ALF system (Pharmacia, Piscataway, NJ).

RESULTS

Stages of Fiber Development. The proteins of 14-DPA fiber cells were compared with those of 21-DPA fiber. To ascertain when the developing fibers began SCW synthesis, the cellulose content of developing fibers was measured (Figure 1). There was a large increase in cellulose in fibers at 20 DPA, indicating the onset of SCW synthesis by this time.

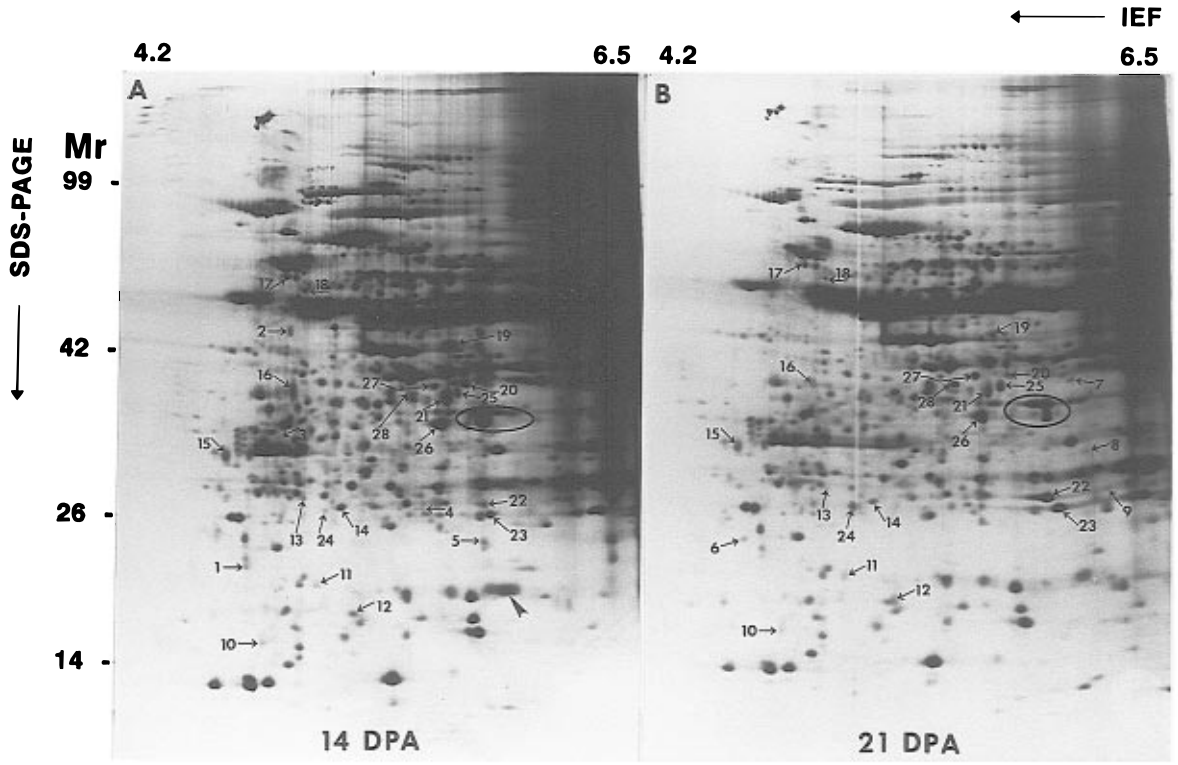

Figure 2. Comparison of the proteins from cotton fiber cells at 14 (A) and 21 DPA (B). Proteins labeled 1−5 (A) were evident only in the 14-DPA gels. Proteins labeled 6−9 (Panel B) were evident only in the 21-DPA gels. Proteins labeled 10−21 were present in higher amounts in the 14-DPA gels. Proteins labeled 22−28 were present in higher amounts in the 21-DPA gels. The arrowhead in panel A indicates a protein that appears to be evident only in the 14-DPA gel; however, this protein was variable and not always present in the 14-DPA gels. Significant differences can be seen in the pattern of proteins within the oval indicated in panels A and B; however, the low resolution in this region did not permit their analysis. Molecular mass markers are shown in kilodaltons.

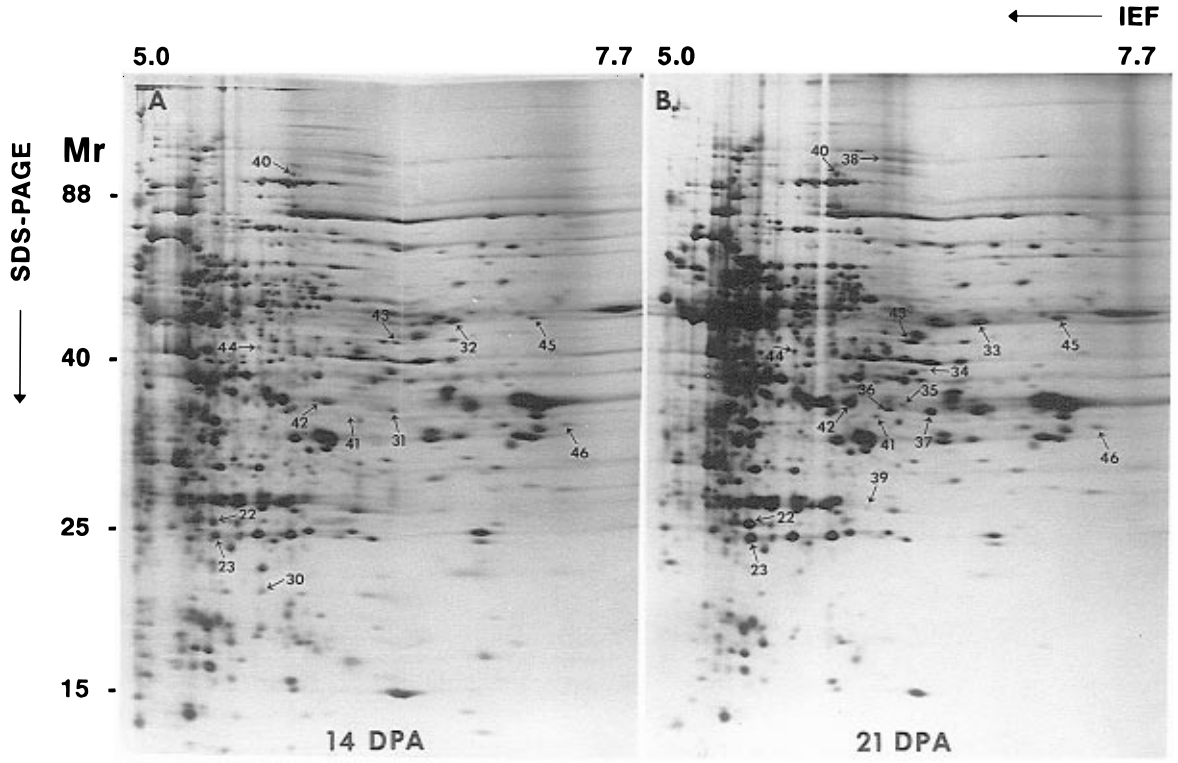

Figure 3. Comparison of the proteins from cotton fiber cells at 14 (A) and 21 DPA (B). Proteins labeled 30−32 (A) were evident only in the 14-DPA gels. Proteins labeled 33−39 (B) were evident only in the 21-DPA gels. Proteins labeled 40−46 were present in higher amounts in the 21-DPA gels. Molecular mass markers are shown in kilodaltons.

2-D PAGE Gels. The 2-D PAGE gels shown in Figure 2 separated proteins with isoelectric points (p*I*) between pH 4.2 and 6.5. Analysis of these gels showed that 95% of the approximately 600 total proteins were equally represented at both 14 and 21 DPA. Twenty-eight proteins, representing 5% of the total proteins,

were either "unique" at one stage or showed <2-fold difference in integrated intensities between the two stages. The legend of Figure 2 describes the numbering for these identified proteins and indicates which proteins increased or decreased between 14 and 21 DPA. A large number of proteins on the basic end of Figure 2 gels were poorly separated; therefore, a second set of gels was run in a higher IEF pH range to better separate these proteins.

This second set of gels is shown in Figure 3, where proteins with p*I* values between pH 5.0 and 7.7 are separated. A subset of proteins separated in the gels of Figure 2 are also present in the gels of Figure 3. For example, proteins 22 and 23 are marked in both Figures 2 and 3. The analysis of Figure 3 gels found that 97% of the approximately 550 proteins were similarly present at both stages. Seventeen proteins, representing 3% of the total proteins, were found to change in quantity between the two stages as indicated in Figure 3.

Protein quantities were measured in relative units because the relationship between the density of silver staining and concentration of protein is unique to each protein (Merril et al., 1984), and we have not attempted to determine this relationship for each protein. However, the integrated intensities are proportional to the density of silver staining for each protein spot. Therefore, the differences in integrated intensities for each protein spot represent actual differences in protein quantity; however, the degree of the difference remains undetermined.

Overall, we have found 45 proteins (Figures 2 and 3) that changed in quantity between the midelongation stage (14 DPA) and the early SCW synthesis stage (21 DPA). Table 1 lists the relative molecular weight (M_r), p*I*, and relative quantities of these proteins.

N-Terminal Protein Sequencing and Analysis. We chose three proteins (proteins 22, 37, and 42) for further study because they were present in greater quantities at 21 DPA than at 14 DPA. These three proteins were analyzed to determine their N-terminal amino acid sequences. Protein 37 had a blocked N terminus, and no sequence was obtained.

A 13 amino acid sequence from the N terminus for protein 22 is Trp-Arg-Ile-Gly-Gln-Asn-Pro-Glu-Phe-Val-Ser-Ile-Lys. Comparison with sequences in the SwissProt and GenBank databases identified a few possible matches, including human β-1,6-*N*-acetylglucosaminyl transferase with 61% identity (Bierhuizen and Fukuda, 1992) and PsaD, a cyanobacterial protein that complexes with ferredoxin and ferredoxin oxidoreductase with 77% identity (Reilly et al., 1988). The large increase in this protein when the fiber cells are beginning SCW synthesis warrants further investigation.

The amino acid sequence of protein 42 is shown in Table 2 along with its alignment with other MDH sequences. Protein 42 had the highest sequence identity to a putative cytoplasmic MDH from *Arabidopsis*. Much lower identities were obtained to MDH from the bacterium *Thermus aquaticus* and mouse (*Mus musculus*) cytoplasmic MDH. In contrast, the sequence similarity between protein 42 and organellar forms of plant MDH is much lower (Table 2).

Table 1. Summary of the Cotton Fiber Proteins Illustrated in Figures 2 and 3 That Differ in Relative Amount between 14- and 21-DPA Stages of Development

			relative amount[a]	
protein no.	M_r	p*I*	14 DPA	21 DPA
		Figure 2		
1	21 900	4.3	+	−
2	45 300	4.7	+	−
3	33 100	4.7	+	−
4	26 500	5.5	+	−
5	23 900	5.8	+	−
6	20 800	4.2	−	+
7	36 600	5.8	−	+
8	29 900	6.0	−	+
9	26 700	6.2	−	+
10	15 300	4.4	++	+
11	20 200	4.9	++	+
12	17 600	5.1	++	+
13	27 200	4.9	++	+
14	26 500	5.1	++	+
15	31 500	4.2	++	+
16	37 700	4.8	+++	+
17	58 200	4.7	++	+
18	54 600	4.8	++	+
19	42 100	5.6	++	+
20	38 300	5.7	++	+
21	36 900	5.6	++	+
22	27 200	5.8	+	+++
23	26 200	5.8	+	++
24	26 200	5.0	+	+++
25	37 400	5.7	+	++
26	34 500	5.6	+	++
27	38 100	5.6	+	++
28	36 800	5.4	+	+++
		Figure 3		
30	21 200	6.1	+	−
31	35 600	6.9	+	−
32	46 400	7.2	++	−
33	46 200	7.2	−	++
34	39 000	7.0	−	+
35	36 200	6.9	−	+
36	35 700	6.9	−	+
37	35 000	7.0	−	+++
38	110 500	6.8	−	+
39	27 000	6.6	−	+
40	100 800	6.5	+	++
41	34 800	6.8	+	++
42	36 500	6.7	+	++
43	42 200	6.9	+	++
44	42 100	6.4	+	++
45	47 300	7.5	+	++
46	34 600	7.6	+	++

[a] +, ++, and +++ indicate relative difference in the integrated intensities of the respective proteins at each stage; − indicates absence of the protein.

DISCUSSION

New information about the biochemistry and physiology of the growth of cotton fiber cells is needed for continued improvement in fiber quality and yield. With respect to this goal, we have chosen to analyze the cellular proteins at two stages in fiber development, 14 and 21 DPA. The cellulose content data in Figure 1 demonstrate that commencement of the SCW begins between 16 and 20 DPA. Fiber harvested at 14 DPA is prior to this period and represents the midelongation stage of development with primary cell wall synthesis. Fiber harvested at 21 DPA represents fiber that has begun SCW synthesis.

With 2-D PAGE, we analyzed approximately 800−900 proteins at each of the two stages. We are not the first group to attempt 2-D PAGE separation of total protein extracted from the fiber: Graves and Stewart (1988) previously published 2-D gels of cotton fiber proteins. No similarity in the protein profiles existed between these previously published gels and the gels presented here. Reasons for this dissimilarity include differences in extraction and isoelectric focusing techniques which resulted in a lower resolution of the previous gels. Graves and Stewart (1988) reported that

Table 2. Alignment of Protein 42 N-Terminal Amino Acid Sequence with Other Malate Dehydrogenase Sequences

source	cellular location	sequence[a]															% identity	ref
protein 42[b]		K	E	P	V	Q	V	L	V	T	G	A	A	G	Q	I	G	100	
Arabidopsis thaliana	cytoplasm	*	*	*	*	R	*	*	*	*	*	*	*	*	*	*	*	94	Newman et al. (1994); this paper
Thermus aquaticus		*	A	*	*	R	*	A	*	*	*	*	*	*	*	*	*	81	Nishiyama et al. (1986)
Mus musculus (mouse)	cytoplasm	S	*	*	I	R	*	*	*	*	*	*	*	*	*	*	A	75	Joh et al. (1987)
Zea mays	chloroplast	R	K	L	*	N	*	A	*	S	*	*	*	*	M	*	S	50	Metzler et al. (1989)
Citrullus vulgaris	mitochondria	V	P	E	R	K	*	A	*	L	*	*	*	*	G	*	*	50	Gietl et al. (1986)
Citrullus vulgaris	glyoxysomes	A	P	G	F	K	*	A	I	L	*	*	*	*	G	*	*	44	Gietl et al. (1986)

[a] Standard one-letter abbreviation of amino acids. Identical residues to the protein 42 sequence are denoted with asterisks (*). [b] The sequence shown starts with the second amino acid; the first amino acid was ambiguous, being either aspartate or alanine.

the difficulties they encountered could be attributed to the presence of polyphenolic compounds present in the cotton tissue. With our protocols using phenol extraction of the proteins, we were able to overcome these past difficulties and obtain good resolution of the proteins with 2-D PAGE.

We were not able to tentatively identify any of the proteins separated on our 2-D PAGE gels on the basis of M_r and p*I*. Dixon et al. (1994) reported that cotton α- and *β*-tubulins have apparent molecular masses of 50 kDa and p*I* values ranging from pH 5 to 6. These proteins should be present on our gels shown in Figure 1; however, the lower resolution of proteins in the 50 kDa region prevents them from being specifically identified. Indeed, the large amount of tubulins in fiber (Kloth, 1989) may partly account for the overstaining in this region. Therefore, without knowing the identities of any of the separated proteins, we compared the differences in protein profiles between the two stages of fiber development.

Of the total proteins analyzed, 45 proteins were identified (Figures 1 and 2) that changed in quantity between 14 and 21 DPA. Twenty proteins were present in greater quantities at 14 DPA, and 25 proteins were present in greater quantities at 21 DPA. Interestingly, we found no massive "expression"/accumulation of specific proteins or protein groups at 21 DPA that could be identified as candidates for the cellulose synthase complex (Okuda et al., 1993), even though this is the time when the large buildup of cellulose in the SCW begins. Likewise, John and Crow (1992) did not find massive differences in the mRNA population isolated during the elongation stage vs the SCW synthesis stage. These results suggest that subtle differences in the cellular protein profiles may account for the changes that occur during fiber development.

We were able to tentatively identify protein 42 as cytoplasmic MDH on the basis of high sequence identity with other cytoplasmic MDH sequences and lower sequence identity with the organellar MDH types. The presence of MDH localized in the cytoplasm in plants has been previously described (Gietl, 1992). No sequence data were available for cytoplasmic MDH in plants until recently reported by Newman et al. (1994). Joh et al. (1987) found that cytoplasmic MDHs were very similar between species; however, the sequence identity between cytoplasmic and mitochondrial MDH within a species was much lower.

MDH isozymes are key enzymes that participate in malate metabolism, and malate is believed to have a central role in fiber growth. Dhindsa et al. (1975) and Basra and Malik (1983) reported that malate significantly increases during fiber elongation and then decreases as fiber elongation slows and SCW synthesis begins. Dhindsa et al. (1975) found malate and potassium contributed up to 50% of the osmotic potential of elongating fiber. Theoretically, the water entering the cell due to the negative osmotic pressure acts as the force behind cell elongation. Two groups have reported conflicting results after measuring total MDH activity during fiber development. Basra and Malik (1983) found that MDH activity peaked during fiber elongation and then dropped to about half the maximum activity by the beginning of SCW synthesis. In contrast, Wäfler and Meier (1994) found that MDH activity increased through fiber elongation to SCW synthesis. Additionally, Wäfler and Meier (1994) measured isocitrate dehydrogenase (IDH), a mitochondrial enzyme, activity, and found it to be fairly constant. They suggested that since IDH activity was fairly constant, the increase in MDH activity was likely due to an increase in the cytoplasmic MDH isozymes. Our results provide further evidence of this possibility. Protein 42, a putative cytoplasmic MDH, was present in a greater quantity at 21 DPA (early SCW synthesis stage) than at 14 DPA (midelongation stage). The increased presence of cytoplasmic MDH at early SCW synthesis stage elicits questions about its role. Since MDH is a reversible enzyme, it is possible that this isozyme participates in the metabolism of malate stored in the vacuole during the elongation stage. This scenario is in agreement with the decrease in malate content during early SCW synthesis as reported by Dhindsa et al. (1975) and Basra and Malik (1983).

The advantage of analyzing a large number of proteins simultaneously with 2-D PAGE was recognized many years ago by O'Farrell (1975), but this method has not been fully utilized in plant cell biology research. Reasons for this include the difficulties associated with adverse side reactions of proteins with secondary metabolites, e.g. polyphenolic compounds, and the difficulties associated with obtaining single cell types in plants. The work reported here takes advantage of the large number of nearly synchronously developing plant cells that can be harvested at selected stages of development from cotton bolls. The identification of 1 of these 45 proteins, protein 42, as a putative cytoplasmic MDH validates the usefulness of 2-D PAGE for examining gene regulation in developing cotton fiber. As the other 44 proteins are characterized, they will reveal additional insights into the complex processes of plant cell expansion and secondary cell wall synthesis.

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LITERATURE CITED

Amor, Y.; Mayer, R.; Benziman, M.; Delmer, D. Evidence for a cyclic diguanylic acid-dependent cellulose synthase in plants. *Plant Cell* **1991**, *3*, 989−995.

Basra, A. S.; Malik, C. P. Dark metabolism of CO_2 during fibre elongation of two cottons differing in fibre lengths. *J. Exp. Bot.* **1983**, *34*, 1−9.

Bierhuizen, M. F. A.; Fukuda, M. Expression cloning of a cDNA encoding UDP-GlcNAc:Galβ1−3GalNAc-R (GlcNAc to GalNAc) β1−6GlcNAc transferase by gene transfer to CHO cells expressing polyoma large tumor antigen. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 9326−9330.

Brutlag, D. L.; Dautrico, J. P.; Maulik, S.; Relph, J. Improved sensitivity of biological sequence database searches. *Comp. Appl. Biol. Sci.* **1990**, *6*, 237−245.

DeLanghe, E. A. L. Lint development. In *Cotton Physiology*; Mauney, J. R., Stewart, J. M., Eds.; The Cotton Foundation: Memphis, TN, 1986; pp 325−349.

Delmer, D. P.; Read, S. M.; Cooper, G. Identification of a receptor protein in cotton fibers for the herbicide 2,6-dichlorobenzonitrile. *Plant Physiol.* **1987**, *84*, 415−420.

Dhindsa, R. S.; Beasley, C. A.; Ting, I. P. Osmoregulation in cotton fiber. Accumulation of potassium and malate during growth. *Plant Physiol.* **1975**, *56*, 394−398.

Dixon, D. C.; Seagull, R. W.; Triplett, B. A. Changes in the accumulation of alpha- and beta-tubulin isotypes during cotton fiber development. *Plant Physiol.* **1994**, *105*, 1347−1353.

Gietl, C. Malate dehydrogenase isoenzymes: cellular locations and role in the flow of metabolites between the cytoplasm and cell organelles. *Biochim. Biophys. Acta* **1992**, *1100*, 217−234.

Gietl, C.; Lottspeich, F.; Hock, B. Sequence homologies between glyoxysomal and mitochondrial malate dehydrogenase. *Planta* **1986**, *169*, 555−558.

Graves, D. A.; Stewart, J. M. Analysis of the protein constituency of developing cotton fibres. *J. Exp. Bot.* **1988**, *39*, 59−69.

Hochstrasser, D. F.; Merril, C. R. "Catalysts" for polyacrylamide gel polymerization and detection of proteins by silver staining. *Appl. Theor. Electrophoresis* **1988**, *1*, 35−40.

Hochstrasser, D. F.; Harrington, M. G.; Hochstrasser, A. C.; Miller, M. J.; Merril, C. R. Methods for increasing the resolution of two-dimensional protein electrophoresis. *Anal. Biochem.* **1988**, *173*, 424−435.

Hurkman, W. J.; Tanaka, C. K. Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. *Plant Physiol.* **1986**, *81*, 802−806.

Jasdanwala, R. T.; Singh, Y. D.; Chinoy, J. J. Auxin metabolism in developing cotton hairs. *J. Exp. Bot.* **1977**, *28*, 1111−1116.

Joh, T.; Takeshima, H.; Tsuzuki, T.; Setoyama, C.; Shimada, K.; Tanase, S.; Kuramitsu, S.; Kagamiyama, H.; Morino, Y. Cloning and sequence analysis of cDNAs encoding mammalian cytosolic malate dehydrogenase. Comparison of the amino acid sequences of mammalian and bacterial malate dehydrogenase. *J. Biol. Chem.* **1987**, *262*, 15127−15131.

John, M. E.; Crow, L. J. Gene expression in cotton (*Gossypium hirsutum* L.) fiber: cloning of the mRNAs. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5769−5773.

Kloth, R. H. Changes in the level of tubulin subunits during development of cotton (*Gossypium hirsutum*) fiber. *Physiol. Plant.* **1989**, *76*, 37−41.

LeGendre, N.; Mansfield, M.; Weiss, A.; Matsudaira, P. Purification of proteins and peptides by SDS-PAGE. In *A Practical Guide to Protein and Peptide Purification for Microsequencing*; Matsudaira, P., Ed.; Academic Press: San Diego, CA, 1993; pp 71−101.

Meinert, M. C.; Delmer, D. P. Changes in biochemical composition of the cell wall of the cotton fiber during development. *Plant Physiol.* **1977**, *59*, 1088−1097.

Meredith, W. R., Jr.; Bridge, R. R. Breakup of linkage blocks in cotton, *Gossypium hirsutum* L. *Crop Sci.* **1971**, *11*, 695−698.

Meredith, W. R.; Culp, T. W.; Robert, K. Q.; Ruppenicker, G. F.; Anthony, W. S.; Williford, J. R. Determining future cotton variety fiber quality objectives. *Textile Res. J.* **1991**, *61*, 715−720.

Merril, C. R.; Goldman, D.; Van Keuren, M. L. Gel protein stains: silver stain. *Methods Enzymol.* **1984,** *104*, 441−447.

Metzler, M. C.; Rothermel, B. A.; Nelson, T. Maize NADP-malate dehydrogenase: cDNA cloning, sequence, and mRNA characterization. *Plant Mol. Biol.* **1989**, *12*, 713−722.

Naithani, S. C.; Rama-Rao, N.; Krishnan, P. N.; Singh, Y. D. Changes in *o*-diphenol oxidase during fibre development in cotton. *Ann. Bot.* **1981**, *48*, 379−385.

Newman, T.; de Bruijn, F. J.; Green, P.; Keegstra, K.; Kende, H.; McIntosh, L.; Ohlrogge, J.; Raikhel, N.; Somerville, S.; Thomashow, M.; Retzel, E.; Somerville, C. Genes galore: a summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. *Plant Physiol.* **1994**, *106*, 1241−1255.

Nishiyama, M.; Matsubara, N.; Yamamoto, K.; Iijima, S.; Uozumi, T.; Beppu, T. Nucleotide sequence of the malate dehydrogenase gene of *Thermus flavus* and its mutation directing an increase in enzyme activity. *J. Biol. Chem.* **1986**, *261*, 14178−14183.

O'Farrell, P. H. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **1975**, *250*, 4007−4021.

O'Farrell, P. Z.; Goodman, H. M.; O'Farrell, P. H. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **1977**, *12*, 1133−1142.

Okuda, K.; Li, L. K.; Kudlicka, K.; Kuga, S.; Brown, R. M. beta-glucan synthesis in the cotton fiber. 1. Identification of beta-1,4-glucan and beta-1,3-glucan synthesized in vitro. *Plant Physiol.* **1993**, *101*, 1131−1142.

Reilly, P.; Hulmes, J. D.; Pan, Y. C. E.; Nelson, N. Molecular cloning and sequencing of *psaD* gene encoding subunit II of photosystem I from the cyanobacterium, *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* **1988**, *263*, 17658−17662.

Scholl, R. L.; Miller, P. A. Genetic association between yield and fiber strength in upland cotton. *Crop Sci.* **1976**, *16*, 780−783.

Schubert, A. M.; Benedict, C. R.; Berlin, J. D.; Kohel, R. J. Cotton fiber development−kinetics of cell elongation and secondary wall thickening. *Crop Sci.* **1973**, *13*, 704−709.

Stewart, J. M. Fiber initiation on the cotton ovule (*Gossypium hirsutum*). *Am. J. Bot.* **1975**, *62*, 723−730.

Updegraff, D. M. Semi-micro determination of cellulose in biological materials. *Anal. Biochem.* **1969**, *32*, 420−424.

Wäfler, U.; Meier, H. Enzyme activities in developing cotton fibres. *Plant Physiol. Biochem.* **1994**, *32*, 697−702.

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